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23S rRNA Gene Mutations Contributing to Macrolide Resistance in *Campylobacter jejuni* and *Campylobacter coli*

Scott R. Ladely,[1] Richard J. Meinersmann,[1] Mark D. Englen,[1]
Paula J. Fedorka-Cray,[1] and Mark A. Harrison[2]

Abstract

The genetic basis of macrolide resistance in *Campylobacter coli* ($n = 17$) and *C. jejuni* ($n = 35$) isolates previously subjected to *in vivo* selective pressure was investigated to determine if the number of copies of 23S rRNA genes with macrolide-associated mutations affects the minimum inhibitory concentration (MIC) of macrolides. Sequence data for domain V of the 23S rRNA gene revealed that two macrolide-resistant *C. coli* isolates had adenine→guanine transitions at position 2059 (A2059G, *Escherichia coli* numbering). One of the two isolates had the A2059G transition in only two of the three gene copies. Among the macrolide-resistant *C. jejuni* isolates ($n = 9$), two different point mutations within domain V were observed. Three macrolide-resistant *C. jejuni* isolates had A2059G transitions. One of these three *C. jejuni* isolates had the A2059G transition in only two of the three gene copies. Six macrolide-resistant *C. jejuni* isolates had an adenine→cytosine transversion at position 2058 (A2058C, *E. coli* numbering) in all three copies of the 23S rRNA gene. *Campylobacter jejuni* isolates with the A2058C transversion had higher erythromycin MICs (>256 μg/mL) compared to *C. jejuni* isolates with A2059G transitions (64–128 μg/mL). In addition, the *C. jejuni* and *C. coli* isolates with only two copies of the 23S rRNA gene having A2059G substitutions had lower macrolide MICs compared to isolates with all three copies of the gene mutated. No isolates were observed having only one copy of the 23S rRNA gene with a mutation. Sequence analysis of ribosomal proteins L4 (*rplD*) and L22 (*rplV*) indicated that ribosomal protein modifications did not contribute to macrolide resistance among the collection of *Campylobacter* examined.

Introduction

Campylobacter is recognized as a major cause of acute bacterial gastroenteritis in humans worldwide (Friedman *et al*., 2000). Within the United States, an estimated 1.5 million cases of human gastroenteritis are attributed to *Campylobacter* infection annually (Samuel *et al*., 2004). *Campylobacter jejuni* and *C. coli* are the species most frequently isolated from cases of human infection, with *C. jejuni* accounting for over 90% of infections, and *C. coli* being identified in most of the remaining cases (Lastovica and Skirrow, 2000). Human *Campylobacter* infection is generally an acute gastrointestinal illness characterized by diarrhea, abdominal cramping, and fever (Karmali and Fleming, 1979; Blaser *et al*., 1983). The majority of cases are mild or self-limiting and antimicrobial therapy is not required. Nonspecific supportive and symptomatic treatment, as for any other gastrointestinal illness, is usually sufficient (McNulty, 1987). However, prolonged duration of illness or altered immune function in some individuals may warrant antimicrobial therapy (Aarestrup and Engberg, 2001; Allos, 2001). The macrolide

[1]Bacterial Epidemiology and Antimicrobial Resistance Research Unit, Richard B. Russell Agricultural Research Center, Agricultural Research Service, U.S. Department of Agriculture, Athens, Georgia.
[2]Department of Food Science and Technology, University of Georgia, Athens, Georgia.

erythromycin (ERY) is a primary treatment option for *Campylobacter* infections in humans, and as such, the development of macrolide-resistance in *Campylobacter* may pose a public health concern.

Macrolides are a class of antimicrobial compounds derived from secondary metabolism products of streptomycetes bacteria (Vester and Douthwaite, 2001). Their structures consist of 14-, 15-, or 16-member lactone rings with two or more amino or neutral sugars attached (Vester and Douthwaite, 2001). These compounds are effective against Gram-positive cocci and bacilli, Gram-negative cocci, and a limited number of Gram-negative bacilli which include *Bordetella pertussis*, *Campylobacter*, *Chlamydia*, *Helicobacter*, and *Legionella* species (Leclercq, 2002).

The basic mechanism of action of macrolides is the inhibition of protein synthesis. During protein synthesis, the macrolide desosamine sugar group forms hydrogen bonds with the polar groups on the 23S rRNA nucleotides 2058 and 2059 (*Escherichia coli* numbering) at the narrowest portion of the 50S subunit tunnel wall. This effectively blocks growth of the nascent peptide chain (Franceschi *et al*., 2004; Poehisgaard and Douthwaite, 2005). With larger macrolide molecules such as tylosin (TYL), the five-disaccharide group extends up the tunnel to the peptidyl-transferase center, directly interfering with peptide-bond formation (Poulsen *et al*., 2000).

Single base substitutions at positions 2058 and 2059 in domain V of 23S rRNA (*E. coli* numbering) have been shown to confer macrolide resistance in *Campylobacter* and several other bacterial genera (Jensen and Aarestrup, 2001; Vester and Douthwaite, 2001). Bacterial species possessing multiple copies of the 23S rRNA gene may require more than one mutated copy to confer macrolide resistance. A mutation in one of the two 23S rRNA copies of *Helicobacter pylori* has been shown to confer macrolide resistance (Hultén *et al*., 1997). While transformation studies utilizing *Streptococcus pneumoniae*, which has four copies of 23S rRNA, have shown that susceptibility to ERY decreases as the number of mutated gene copies increases (Tait-Kamradt *et al*., 2000). Macrolide-associated mutations in a single copy of the three *Campylobacter* 23S rRNA genes have not been reported, nor has there been any correlation of the number of mutated target gene copies with levels of macrolide resistance.

In this study, the genetic basis of macrolide resistance in a collection of *C. coli* and *C. jejuni* isolates previously subjected to *in vivo* selective pressure was investigated to determine if the number of copies of 23S rRNA genes with macrolide-associated mutations affects the minimum inhibitory concentration (MIC) of three macrolides or a lincosamide. The macrolide-susceptible parent strains used in this study were confirmed to have wild-type 23S rRNA genes by sequence analysis of domain V for each of the three gene copies. It was hypothesized that the MICs of derived strains would be proportional to the number of mutated gene copies.

Materials and Methods

Campylobacter *isolates*

Campylobacter isolates used in this study were obtained from chicken ceca (17 *C. coli* and 35 *C. jejuni*), as part of a previous study in which broilers were challenged with macrolide-susceptible strains of *Campylobacter* (three strains of *C. jejuni* or three strains of *C. coli*) and then administered TYL at subtherapeutic or therapeutic concentrations (Ladely *et al*., 2007). All isolates had previously been tested for susceptibility to ERY using the agar dilution method (CLSI, 2006). Approximately 79% of the isolates used in this study were susceptible to macrolides, even though they had been recovered from broilers administered TYL. The susceptible isolates were included in the study to increase the probability of identifying strains with less than three mutated copies of the 23S rRNA gene.

Antimicrobial susceptibility testing

For each isolate, the MICs to three macrolides (azithromycin [AZM], ERY, and TYL) and a lincosamide (clindamycin [CLI]) were determined using the agar dilution method recommended by the Clinical and Laboratory Standards Institute (CLSI, 2006). Isolates were considered resistant to AZM, ERY, and CLI with MICs ≥ 8, ≥ 32, and $\geq 8\,\mu g/mL$, respectively. Interpretive criteria for TYL susceptibility testing have not been established (CLSI, 2006).

Table 1. Primers Used for Polymerase Chain Reaction Amplification and Sequencing

Name	*Sequence (5′–3′)*	*Amplicon size*	*Reference*
23S rRNA gene amplification			
FI	CCCTAAGTCAAGCCTTTCAATCC	5.7 kbp	Gibreel *et al*. 2005
FII	CGTTATAGATACGCTTAGCGGTTATG	5.8 kbp	Gibreel *et al*., 2005
FIII	CATCGAGCAAGAGTTTATGCAAGC	5.7 kbp	Gibreel *et al*., 2005
FIa[a]	TGGCAACGGGCGGAACTA	6.3 kbp	This study
FIIa	CAACAGGGGAAAACGCTTTGATT	6.7 kbp	This study
FIIIa	CCACCAAAAATAAGCCCGTGAA	6.4 kbp	This study
CJ-copy-R	CTACCCACCAGACATTGTCCCAC		Gibreel *et al*., 2005
Sequence fragment, domain V of the 23S rRNA gene			
F1-campy-23S	AAGAGGATGTATAGGGTGTGACG	508 bp	Vacher *et al*., 2003
R1-campy-23S	AACGATTTCCAACCGTTCTG		Vacher *et al*., 2003
L4 ribosomal protein			
L4 Fwd	GTAGTTAAAGGTGCAGTACCA	766 bp	Cagliero *et al*., 2006
L4 Rev	GCGAAGTTTGAATAACTACG		Cagliero *et al*., 2006
L22 ribosomal protein			
L22C-F	TTAGCTTTCCTTTTTCACTGTTGCTTT	425 bp	Corcoran *et al*., 2006
L22C-R	ATGAGTAAAGCATTAATTAAATTCATAAG		Corcoran *et al*., 2006

[a]FIa, FIIa, and FIIIa primers were based on the partially sequenced *C. coli* strain RM2228 (Fouts *et al*., 2005).

Campylobacter jejuni ATCC 33560 was used as a quality control strain for susceptibility testing.

Polymerase chain reaction (PCR) amplification and DNA sequencing

The primers used for amplification and sequencing of domain V of the 23S rRNA gene and the *rplD* and *rplV* genes that encode the L4 and L22 ribosomal proteins, respectively, are listed in Table 1. Genomic DNA for PCR was prepared using the Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN) according to manufacturer's directions. Primers flanking each operon were utilized to amplify the three copies of the 23S rRNA gene in all *C. coli* and *C. jejuni* isolates. *Campylobacter jejuni* amplifications were performed in three separate reactions as described by Gibreel *et al*. (2005), using forward primers FI, FII, or FIII, paired with the conserved reverse primer CJ-copy-R. Similarly, *C. coli* amplifications were performed using forward primers FIa, FIIa, or FIIIa, paired with reverse primer CJ-copy-R. Potential macrolide-associated mutations were then identified by sequencing a 508-bp fragment (Vacher *et al*., 2003) for each copy of the target gene.

To assess the contribution of mutations within L4 and L22 ribosomal protein genes (*rplD* and *rplV*, respectively) to macrolide resistance, sequence analysis of L4 and L22 ribosomal protein genes of macrolide-resistant *Campylobacter* strains (*C. coli* $n = 2$, *C. jejuni* $n = 9$) and their macrolide-susceptible parent strains were compared. PCR amplifications of *rplD* and *rplV* genes were performed as describe by Cagliero *et al*. (2006) and Corcoran *et al*. (2006), respectively. PCR products were analyzed by gel electrophoresis, purified using the QIAquick PCR purification system (Qiagen, Valencia, CA) and were sequenced using the BigDye Terminator 1.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA) and an ABI 3100 Genetic Analyzer (Applied Biosystems). Forward and reverse sequence data were assembled and compared using Sequencher version 4.2 (Gene Codes Corporation, Ann Arbor, MI).

Results

Resistance patterns of Campylobacter *isolates*

The MICs to the three macrolides (AZM, ERY, and TYL) and a lincosamide (CLI) were determined for 17 *C. coli* (Table 2) and 35 *C. jejuni* isolates (Table 3) using agar dilution. Eighty-eight percent (15/17) of the *C. coli* and 74% (26/35) of the *C. jejuni* isolates were susceptible to AZM, ERY, and CLI. Two *C. coli* isolates (TPS61 and TSP62) were resistant to AZM and ERY; one (TPS61) was also resistant to CLI. Nine *C. jejuni* isolates (26%) were resistant to AZM,

Table 2. Minimum Inhibitory Concentrations (MICs) for *Campylobacter coli* Isolates and Corresponding Resistance-Associated Mutation in the 23S rRNA Gene

	MIC (μg/mL)[a]				
Isolate	*AZM*	*ERY*	*CLI*	*TYL*	*23S rRNA gene mutation*[b]
TPS13	1	1	1	2	Wild-type
TPS14	1	1	1	2	Wild-type
TPS15	1	1	1	2	Wild-type
TSP16	1	1	1	2	Wild-type
TPS03	1	1	1	4	Wild-type
TPS05	1	1	1	4	Wild-type
TPS06	1	1	1	4	Wild-type
TPS07	1	1	1	4	Wild-type
TSP08	1	1	1	4	Wild-type
TPS10	1	1	1	4	Wild-type
TPS04	1	1	1	8	Wild-type
TPS09	1	1	1	8	Wild-type
TPS11	1	1	1	8	Wild-type
TSP01	1	4	1	32	Wild-type
TSP02	1	4	1	32	Wild-type
TPS62	256	32	2	4	A2059G[c]
TPS61	>256	64	8	64	A2059G

[a]Isolates were considered resistant to azithromycin (AZM), erythromycin (ERY), and clindamycin (CLI) with MICs ≥8, ≥32, and ≥8 μg/mL, respectively. The interpretive criteria for tylosin (TYL) susceptibility testing have not been established (CLSI, 2006).
[b]The position of the 23S rRNA gene mutation is based on the numbering of the *E. coli* gene. Unless otherwise indicated, data is for all three copies of the 23S rRNA gene.
[c]The mutation in this isolate was detected in only two copies of the 23S rRNA gene.

Table 3. Minimum Inhibitory Concentrations (MICs) for *Campylobacter jejuni* Isolates and Corresponding Resistance-Associated Mutation in the 23S rRNA Gene

	MIC (μg/mL)[a]				
Isolate	*AZM*	*ERY*	*CLI*	*TYL*	*23S rRNA gene mutation*[b]
33560[c]	1	1	1	2	Wild-type
TPS36	1	1	1	2	Wild-type
TPS20	1	1	1	4	Wild-type
TPS25	1	1	1	4	Wild-type
TPS26	1	1	1	4	Wild-type
TPS27	1	1	1	4	Wild-type
TPS28	1	1	1	4	Wild-type
TPS29	1	1	1	4	Wild-type
TPS30	1	1	1	4	Wild-type
TPS31	1	1	1	2	Wild-type
TPS33	1	1	1	4	Wild-type
TPS34	1	1	1	4	Wild-type
TPS35	1	1	1	4	Wild-type
TPS39	1	1	1	4	Wild-type
TPS41	1	1	1	4	Wild-type
TPS21	1	1	1	8	Wild-type
TPS22	1	1	1	8	Wild-type
TPS23	1	1	1	8	Wild-type
TPS37	1	1	1	8	Wild-type
TPS19	1	1	1	16	Wild-type
TPS40	1	2	1	16	Wild-type
TPS60	1	2	2	16	Wild-type
TPS24	1	4	1	16	Wild-type
TPS38	1	4	1	16	Wild-type
TPS57	1	4	1	32	Wild-type
TPS32	1	8	1	32	Wild-type
TPS59	1	8	1	32	Wild-type
TPS53	256	64	4	32	A2059G[d]
TPS49	>256	128	8	64	A2059G
TPS50	>256	128	8	64	A2059G
TPS48	>256	>256	8	64	A2058C
TPS42	>256	>256	16	>256	A2058C
TPS43	>256	>256	16	>256	A2058C
TPS45	>256	>256	16	>256	A2058C
TPS46	>256	>256	16	>256	A2058C
TPS47	>256	>256	16	>256	A2058C

[a]Isolates were considered resistant to azithromycin (AZM), erythromycin (ERY), and clindamycin (CLI) with MICs ≥8, ≥32, and ≥8 μg/mL, respectively. The interpretive criteria for tylosin (TYL) susceptibility testing have not been established (CLSI, 2006).
[b]The position of the 23S rRNA gene mutation is based on the numbering of the *E. coli* gene. Unless otherwise indicated, data is for all three copies of the 23S rRNA gene.
[c]*C. jejuni* strain ATCC 33560 was included as a representative of macrolide-susceptible strains.
[d]The mutation in this isolate was detected in only two copies of the 23S rRNA gene.

ERY, and CLI (Table 3). The TYL MICs for *C. coli* and *C. jejuni* isolates ranged from 2 to >256 μg/mL.

Sequence analysis of 23S rRNA and L4 and L22 ribosomal protein genes

Operon-specific PCRs were performed to amplify the three copies of the 23S rRNA gene for each isolate. The resulting PCR products were all of the expected sizes. Sequencing data for domain V of the 23S rRNA gene showed that all macrolide-susceptible *C. coli* and *C. jejuni* isolates had nonmutated wild-type 23S rRNA. None of the isolates, susceptible or resistant, were found to have only one mutated copy of the 23S rRNA gene.

The two macrolide-resistant *C. coli* isolates (TPS61 and TPS62) had adenine→guanine transitions at position 2059 (A2059G, *E. coli* numbering) of the 23S rRNA gene (Table 2). One of the macrolide-resistant *C. coli* isolates (TPS61) had A2059G transitions in all three target genes, the other (TPS62) had the A2059G transition in only two of the three copies of the 23S rRNA gene.

Among the nine macrolide-resistant *C. jejuni* isolates, two different point mutations within domain V of the 23S rRNA gene were observed

(Table 3). Two resistant *C. jejuni* isolates (TPS49 and TPS50) exhibited the A2059G transition in all three 23S rRNA gene copies, while one *C. jejuni* isolate (TPS53) had A2059G transitions in only two of the three copies of the target gene. The remaining six of these nine macrolide-resistant *C. jejuni* isolates (TPS42, TPS43, TPS45, TPS46, TPS47, and TPS48) had an adenine→cytosine transversion at position 2058 (A2058C) in all three copies of the 23S rRNA gene.

The six *C. jejuni* isolates (TPS42, TPS43, TPS45, TPS46, TPS47, and TPS48) with the A2058C transversion had higher ERY MICs (>256 μg/mL) compared to the three *C. jejuni* isolates (TPS49, TPS50, and TPS53) with A2059G transitions (64–128 μg/mL). In addition, *C. jejuni* (TPS53) and *C. coli* (TPS62) isolates with only two copies of the 23S rRNA gene having the A2059G substitution had lower ERY, AZM, and TYL MICs compared to isolates in which all three copies of the target gene were mutated (Tables 2 and 3).

Sequence analysis of L4 and L22 ribosomal protein genes of macrolide-resistant *Campylobacter* strains ($n = 2$ *C. coli*, $n = 9$ *C. jejuni*) and their macrolide-susceptible parent strains were compared. Complete DNA sequence identity was observed between macrolide-resistant strains and their susceptible parent strains.

Discussion

Domain V of each of the three copies of 23S rRNA gene were sequenced for 41 macrolide-susceptible (15 *C. coli* and 26 *C. jejuni*) and 11 macrolide-resistant *Campylobacter* isolates (two *C. coli* and nine *C. jejuni*). Sequencing data showed that all macrolide-susceptible *C. coli* and *C. jejuni* isolates had wild-type 23S rRNA. No isolates recovered from poultry with known exposure to TYL, regardless of whether they were resistant, were found to have only one mutated copy of the 23S rRNA gene. No previous reports in which individual operons were sequenced, have identified *Campylobacter* strains with only one copy of the 23S rRNA gene mutated. Further genetic characterizations of susceptible and low-level macrolide-resistant *Campylobacter* strains may identify strains with only a single mutated copy of the 23S rRNA gene. However, since the ancestral state of the study isolates was known to have all three 23S rRNA genes with wild-type alleles, conversion to the second mutated copy of this gene must be extremely rapid, suggesting selective pressure against strains with just one mutant copy. The resistance profile of the isolates with two mutated copies of the 23S rRNA implies that conversion of the third copy is not necessary for fitness in the presence of AZM but may be needed for TYL and/or ERY. No conclusions on the rate of conversion of the third copy can be made and further studies are warranted.

Mutations were identified at positions 2058 or 2059 (*E. coli* numbering system) in all high-level ERY-resistant isolates, as previously reported (Jensen and Aarestrup, 2001; Payot *et al*., 2004; Alonso *et al*., 2005; Corcoran *et al*., 2006). These mutations also provided resistance to AZM and in most cases conferred cross-resistance to the lincosamide CLI, which is also consistent with earlier studies (Taylor and Chang, 1991; Cagliero *et al*., 2005; Mamelli *et al*., 2005). Limited data are available regarding TYL MICs in *Campylobacter* species. Cagliero *et al*. (2005), reported TYL MICs of 32 μg/mL for wild-type *C. coli* and MICs of ≥2048 μg/mL for *C. coli* isolates with A2059G transitions in all three copies of the 23S rRNA gene. TYL MICs in the present study tended to be lower, in part due to a narrower range of testing (1–256 μg/mL). However, both studies found TYL MICs to be 2–32 fold higher than ERY and AZM MICs among wild-type *C. coli* isolates. Interestingly, one macrolide-resistant *C. coli* isolate (TPS62), with the A2059G mutation in two of the three 23S rRNA gene copies had a TYL MIC similar to wild-type isolates (4 μg/mL).

Five of the eleven *Campylobacter* isolates with point mutations in domain V of the 23S rRNA exhibited the A2059G transition, which has been the most common mutation identified among macrolide-resistant *Campylobacter* isolates (Jensen and Aarestrup, 2001; Alonso *et al*., 2005; Corcoran *et al*., 2006). One *C. coli* (TPS53) and one *C. jejuni* isolate (TPS62) were found to have an A2059G transition in only two copies of the 23S rRNA gene, and previous studies have also reported this genotype at a low frequency (Jensen and Aarestrup, 2001; Payot *et al*., 2004; Gibreel *et al*., 2005; Vacher *et al*., 2005). In the present study, the isolates with two copies of the

mutated target gene had lower levels of ERY resistance compared to isolates of the same *Campylobacter* species having all three copies mutated. It was observed in previous studies (Payot *et al*., 2004; Gibreel *et al*., 2007) that *Campylobacter* isolates with the same point mutation (A2059G) in two copies of the 23S rRNA gene had lower ERY MICs compared to isolates carrying mutations in all three copies of the target gene. Similarly, Vacher *et al*. (2005), observed one *C. jejuni* isolate with an A2059T transversion in only two copies of the 23S rRNA gene that had a lower ERY MIC (8 μg/mL) compared to isolates with mutations in all three copies of the target gene. Similar observations have been noted in other bacterial species. Transformation studies utilizing *Streptococcus pneumoniae*, which has four copies of 23S rRNA, have shown that the level of ERY resistance increases as the number of mutated 23S rRNA gene copies increases (Tait-Kamradt *et al*., 2000). In contrast, Gibreel *et al*. (2005), observed no difference in *Campylobacter* ERY MICs with regard to the number of mutated copies of the target gene.

An A2058C transversion was identified in six *C. jejuni* isolates in our study. This mutation was recently identified among a collection of *Campylobacter* isolates by Vacher *et al*., (2003) at a low frequency (2%). Across bacterial species, transversional substitutions (pyrimidine→purine or purine→pyrimidine) such as the A2058C seen here, generally occur at a much lower frequency than transitional substitutions (pyrimidine→pyrimidine or purine→purine) (Li, 1997) such as the A2059G observed in most 23S rRNA mutations. The higher frequency of A2058C transversions observed in this study may be an artifact of the limited genetic diversity among the *Campylobacter* strains used (derived from *Campylobacter*-challenged poultry). Similarly, Lin *et al*. (2007) observed A2058G transitions in *in vivo* selected mutants, in contrast to the more commonly observed A2059G mutation. Both studies suggest that genetic features of a given strain may influence the specific point mutation observed.

Campylobacter jejuni isolates with the A2058C transversion had consistently higher ERY MICs compared to those with A2059G transitions. Differences in the level of macrolide resistance have been shown to be dependent on the position of base substitution in other bacterial species. For example, in *H. pylori*, base substitutions at position 2058 have been shown to confer higher levels of macrolide resistance than similar base substitutions at position 2059 (Wang and Taylor, 1993). Furthermore, in *H. pylori* A2058C transversions confer similar levels of macrolide resistance to A2058G substitutions (Wang and Taylor, 1993). This may suggest that base substitutions at position 2058 are more effective at disrupting macrolide binding. In contrast, a previously reported A2058T transversion in *C. jejuni* (Vacher *et al*., 2005) had a lower ERY MIC than the other mutations detected (A2058C and A2059G). However, in that case the A2058T transversion was only present in two copies of the target gene.

Other resistance mechanisms, in particular efflux systems have previously been shown to provide low-level macrolide-resistance in *Campylobacter* (Payot *et al*., 2004; Corcoran *et al*., 2006). Gibreel *et al*. (2007), suggest that efflux systems may act synergistically with A2059G mutations, and Cagliero *et al*. (2006), indicate that modifications in ribosomal proteins L4 and L22 may act synergistically with the CmeABC efflux system in conferring macrolide resistance. We did not evaluate the efflux systems in our isolates, however, previous susceptibility testing showed that the ancestral strains used were all susceptible to ERY (Ladely *et al*., 2007) and no highly resistant (ERY MICs >32 μg/mL) isolates were found without changes in at least two 23S rRNA genes. Investigation of ribosomal proteins L4 and L22 by sequence analysis of macrolide-resistant strains and their susceptible parent strains indicated that ribosomal protein modifications did not contribute to macrolide resistance among the collection of *Campylobacter* isolated we examined. Other mechanisms conferring macrolide resistance such as methylation of the drug-binding site and drug inactivation have not yet been observed in *Campylobacter* (Yan and Taylor, 1991; Gibreel *et al*., 2005; Corcoran *et al*., 2006).

In conclusion, these data show that A2058C transversions (*E. coli* numbering) in the *C. jejuni* isolates studied exhibit higher ERY MICs compared to *C. jejuni* isolates with A2059G transitions, indicating that base substitution position may influence macrolide resistance levels in this

pathogen. The number of copies of the 23S rRNA gene carrying mutations may also influence the level of resistance, as A2059G transitions in only two copies of this gene conferred lower macrolide MICs than *C. coli* and *C. jejuni* isolates with mutations in all three copies of the target gene. Even though the majority of strains used in this study were derived from *Campylobacter*-challenged poultry with known exposure to macrolides, no *Campylobacter* strains were identified with only one copy of the 23S rRNA gene mutated. Conversion to the second mutated copy appears to be extremely rapid, suggesting selective pressure against strains with just one mutated copy.

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Disclosure Statement

No competing financial interests exist.

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Address reprint requests to:
Richard J. Meinersmann, V.M.D., Ph.D.
Bacterial Epidemiology and Antimicrobial Resistance Research Unit
Russell Agricultural Research Center
Agricultural Research Service
U.S. Department of Agriculture
950 College Station Road
Athens, GA 30605-2720

E-mail: Rick.Meinersmann@ars.usda.gov